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Serological studies on heat-induced interactions of α-lactalbumin and milk proteins

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Summary. The heat denaturation of α-lactalbumin (α-la) in NaCl and KCl solutions, milk ultrafiltrate and milk was studied using the method of micro complement fixation. It was established that this protein was very resistant to heat denaturation and that it was more stable in milk ultrafiltrate than in the other media studied at temperatures up to 70 °C. Of the various milk proteins added to α-la, only β-lactoglobulin (β-lg) formed a heat-induced complex with this protein. This complex was identical in milk ultrafiltrate or in milk and depended on the molar ratio between both proteins; it was not modified by any other milk proteins. The binding of α-la to β-lg changed the ability of the latter protein to bind κ-casein.

The heat-induced interactions between β-lactoglobulin (β-lg) and casein have been one of the predominant subjects investigated in dairy chemistry during the last 20 years. In contrast, only a limited number of studies has been devoted to heat-induced complex formation between α-lactalbumin (α-la) and other milk proteins. Hunziker & Tarassuk (1965), however, were able to demonstrate that an interaction occurred between α-la and β-lg when the proteins were heated together in a buffer at pH 6·7. It should be emphasized that most of the physico-chemical analyses performed on heat-treated milk proteins have been done in buffers, at protein concentrations often different from those found in milk; also it has been uncertain whether results so obtained could be compared to reactions in the original milk medium. Therefore, in the present study, we have compared the immunochemical behaviour of α-la in milk and milk ultrafiltrate, as well as in isolated systems after heat treatment.

MATERIALS AND METHODS

Reagents

α-Lactalbumin. α-Lactalbumin was prepared from bulk raw milk by gel filtration and ion-exchange chromatography by the method of Armstrong *et al.* (1970). Only the highest of the 3 peaks eluted from DEAE-Sephadex was used. The homogeneity of the preparation was checked by polyacrylamide-gel (PAG) disc electrophoresis and by immunoelectrophoresis using a pool of antisera against the whey proteins. The amino acid composition obtained by amino acid analysis was the same as that published by Gordon & Ziegler (1955); a mol. wt of 14176 was chosen for the calculation of molar concentration (Brew, Vanaman & Hill, 1967; corrected by Gordon, 1971).

β-Lactoglobulin. β-Lactoglobulin (Batch 1545 7607, B.D.H. Chemicals Ltd, Poole, England) was further purified by gel filtration on Sephadex G-100. The mean peak eluted (about 98 % of the protein applied to the column) contained equal amounts of both phenotypes A and B, as determined by disc electrophoresis on PAG. The optical density of the coloured bands obtained after staining was measured using a densitometer, type Integraph-CH (Bender and Hobein, Zürich, Switzerland). As most of the β-lg in milk is in the form of the dimer a mol. wt of 36000 was chosen for the calculation of molar concentrations.

Lactoferrin. Lactoferrin was purified from bovine colostrum by precipitation with ammonium sulphate followed by ion-exchange chromatography, first on Sephadex DEAE A-50, and then on Sephadex CM C-50. Lactoferrin was eluted from the cation exchanger with a NaCl concentration gradient. The protein thus obtained had a value of at least 1·35 for the ratio of the optical densities at 460 and 410 nm.

Caseins. Whole casein was prepared from bulk raw milk by isoelectric precipitation (Rüegg, Lüscher & Blanc, 1974). κ-Casein was isolated according to the method of McKenzie & Wake (1961).

Other proteins. Bovine serum albumin, electrophoretic purity 100 %, and bovine gamma globulins were purchased from Behring Werke (Marburg, West Germany) and used without further purification.

Chemicals. Sephadex G-100, CM-Sephadex C-50 and DEAE-Sephadex A-50 were purchased from Pharmacia (Uppsala, Sweden). Ultrogel AcA 54 was obtained from LKB (Bromma, Sweden). Agarose (Indubiose A-37) for Ouchterlony double diffusion was purchased from l'Industriebiologique française, (Gennevilliers, France). Acrylamide for disc electrophoresis was obtained from Serva, Feinbiochemica, (Heidelberg, West Germany).

Milk ultrafiltrate (MUF) was obtained by filtration of bulk raw skim-milk on a Sartorius membrane with a pore diam. of 500 nm (Nr SM 121-36-100, Sartorius membrane-Filter, Göttingen, West Germany), under a pressure of 1·4 MPa of N_2. The solution obtained was kept under refrigeration until use; its chemical composition was as follows (% w/w): Ca, 0·037; P, 0·035; K, 0·103; Na, 0·031; Cl, 0·093; lactose, 4·66; protein, none; total, N, 0·028; non-protein N, 0·028; dry matter, 5·80.

Determination of protein concentrations

Protein concentrations were determined spectrophotometrically by measuring the optical absorbance with a Zeiss Model PM Q II photometer (Carl Zeiss A. G., Zürich, Switzerland). The extinction coefficients used were $E^{1\%}_{280} = 20$ (Kronman & Andreotti, 1964) for α-la and $E^{1\%}_{278} = 9{\cdot}3$ for β-lg. A value of 9·2 was reported by Visser *et al.* (1972) but in our hands the method gave 9·3.

Antibody production

Antibodies to α-la and β-lg were obtained in rabbits by injecting, into both footpads, 1·0 mg protein (1 ml), emulsified with an equal volume of Freund's complete adjuvant (Difco). Four weeks later, an intravenous injection of 3·0 mg protein was given, followed by 2 other injections fortnightly. The rabbits, 5 for each antigen, were bled 4 d after the last immunization, and the antisera pooled.

Determination of residual serological activity of α-lactalbumin

Measurements of conformational changes and quantitation of uncomplexed α-la were performed by the immunological method of micro complement fixation, as described by Levine (1967). This technique, in contrast to the various immunodiffusion methods so much used in dairy chemistry, allows one to determine the degree of complementarity between a partly denatured antigen and its specific antibody, in a pure solution as well as in a complex medium, e.g. milk. Application of this method requires a constant concentration of antiserum and varying quantities of antigen, giving curves for the amounts of complement fixed which resemble quantitative precipitin curves. The high sensitivity of the reaction is due to the use of low concentrations of reagents (antigen, antibody, complement and sensitized erythrocytes). In our experiments, the equivalence point (corresponding to the peak of the curve) of the reaction between α-la and its specific antibody was obtained at a milk dilution of 1 in 25000, using a pool of 5 antisera diluted 6500 times. The peak of the complement fixation curve thus corresponds to a concentration of α-la of about 0·04 μg/ml. In each series of experiments, a reaction was performed with the native protein as antigen (unheated milk, for example), and the complement fixation curve was then used as a reference in calculations of the changes occurring to the curves found with heat-treated antigen. These changes can be of 2 types: a decrease in the amount of the complement fixed at the same antigen concentration as required for maximum fixation (vertical shift), or an increase in the amount of antigen required for maximum fixation (lateral shift). It has been demonstrated that a vertical shift corresponds to a slight structural modification of the antigen, involving only one or a very few antigenic determinants; a more general conformational change would be indicated by a lateral shift which can also be accompanied by a vertical shift (Levine, 1967; Levine & Van Vunakis, 1967).

Double immunodiffusion was performed according to the procedure of Ouchterlony (1967) in 1·0 % agarose, containing 0·9 % NaCl.

Heat treatments

In order to keep constant the experimental conditions throughout this study, 3 batches of α-la were pooled and dialysed against distilled water. After adjusting the concentration of protein to 0·1 % (w/v), 1·0 ml samples were lyophilized in ampoules and stored at 2 °C until use. For each experiment, the protein was dissolved in the necessary amount of solution (see Fig. 1) and the stoppered ampoule heated by immersion in either a water-bath (0–45 °C) or an oil-bath (45–90 °C) maintained at the required temperature ± 0·3 °C. The duration of heating was measured from the time the desired temperature was reached. For kinetic experiments, 5 μl samples were taken at different times during heating and cooled rapidly by dilution with the cold isotonic tris buffer containing 0·1 % (w/v) bovine serum albumin also used for complement fixation.

Iodination

For iodination of α-la with ^{125}I (The Radiochemical Centre, Amersham, England), the method of Hunter (1967) was applied. The amount of chloramine-T used without

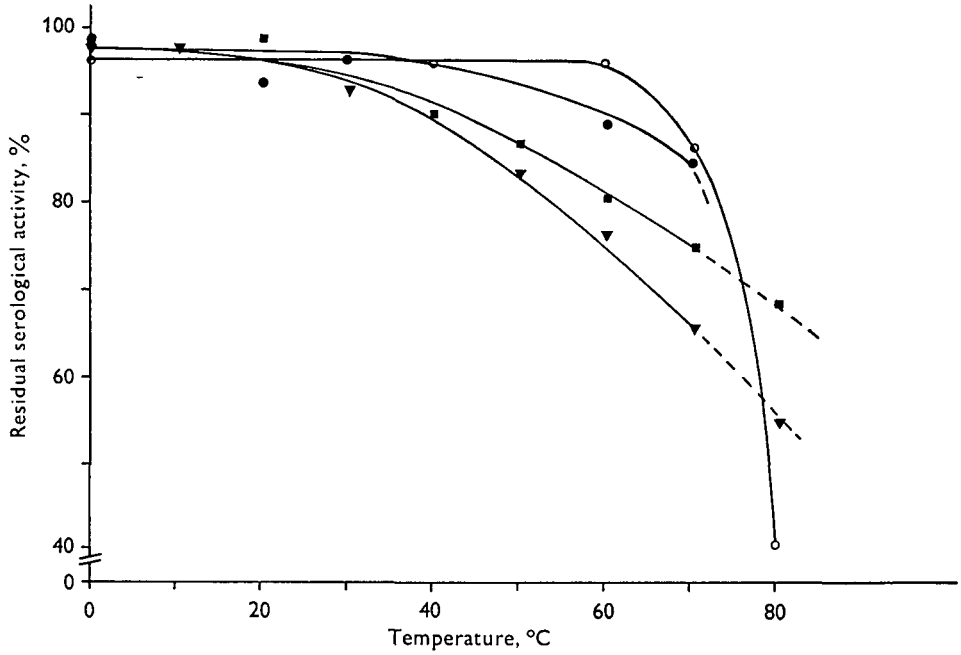

Fig. 1. Stability of α-lactalbumin (α-la) to 3 h incubation at various temperatures in different solutions. The protein was incubated for 180 min at the indicated temperatures, then diluted with buffer at 0 °C and the complement fixation assay performed. The maximum of complement fixation was determined at an antigen concentration of 0·04 μg/ml, under our conditions. ●, α-La tested in milk; ○, in milk ultrafiltrate; ▼, in 0·15 M-NaCl; ■, in 0·15 M-KCl.

damaging the protein was previously determined by several cold-iodinations and the labelled antigen checked for its immunological integrity by the method of micro complement fixation. Radioactivity was measured with a Packard Tri-Carb Liquid scintillation spectrometer, Model 3380 (Packard Instrument International S.A., Zürich, Switzerland).

RESULTS AND DISCUSSION

Heat denaturation of α-lactalbumin

α-Lactalbumin was incubated for 3 h at various temperatures in different solutions, at a concentration of 1·0 mg/ml, and loss of serological activity measured. This concentration was used throughout the study; it corresponds to the concentration of this protein in milk, as measured in several bulk raw milks by the method of micro complement fixation. The effect of solvent on stability to 3 h incubation at various temperatures is presented in Fig. 1. It can be seen that α-la dissolved in MUF could be heated to 60 °C without losing any antigenic activity, whereas some of this activity was already lost at 40 °C when the protein was diluted in 0·15 M-NaCl or 0·15 M-KCl, since there was a decrease in complement fixation at the same antigen concentration as required for maximum fixation with unheated α-la. At 70 °C, a marked change in antigenicity was observed with each solution; at 80 °C, a drastic conformational change of α-la appeared, as one needs more antigen to obtain the equivalence point of the immunological reaction, producing a lateral shift of the

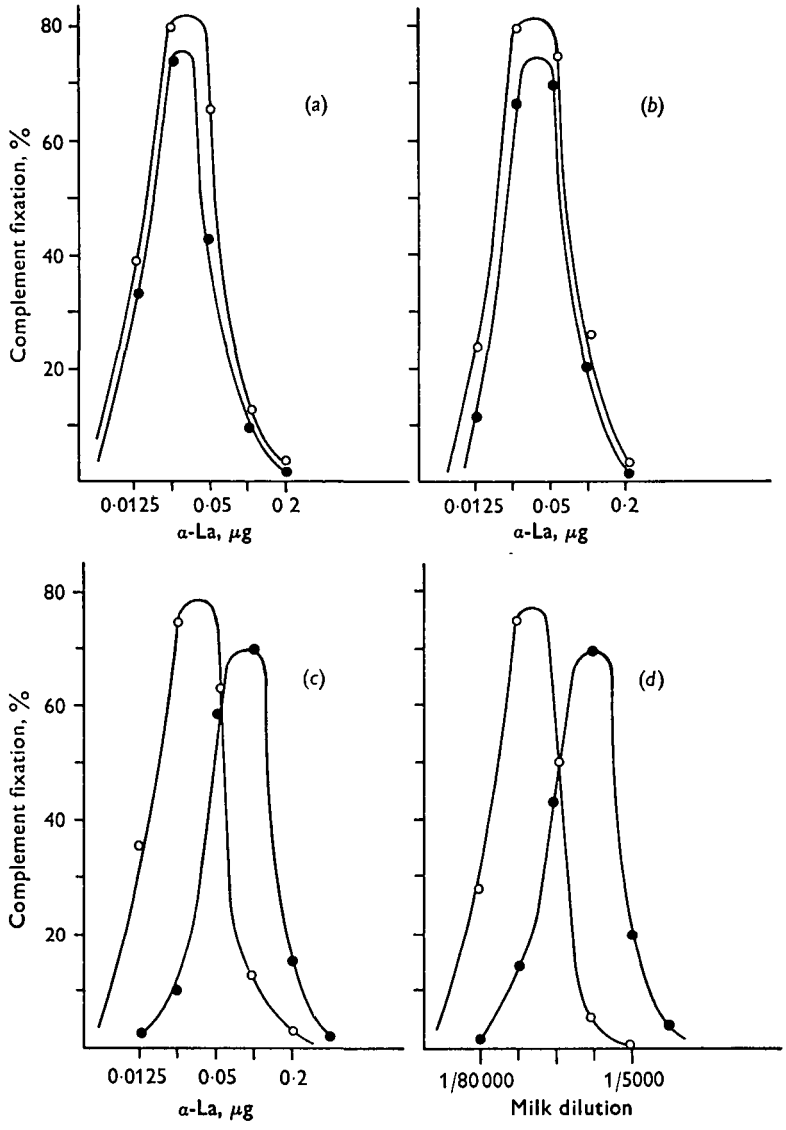

Fig. 2. Influence of various milk proteins on the serological properties of α-lactalbumin (α-la). The different mixtures, dissolved in milk ultrafiltrate, were heated at 80 °C for 30 min. (*a*) α-La alone, (*b*) α-la with immunoglobulins, bovine serum albumin, lactoferrin and whole casein, (*c*) α-la with β-lactoglobulin, (*d*) raw milk. The concentrations of the various proteins were the same as those in milk. ○, unheated α-la [unheated milk for (*d*)]; ●, heat-treated α-la [heat-treated milk for (*d*)].

curve of complement fixation (the dotted lines on Fig. 1 indicate values measured for concentration of 0·04 μg/ml antigen, although the peak of the curve was obtained at a concentration of 0·08 μg/ml). However, this peak shift did not appear when the protein was dissolved in MUF, whereas the shift was more pronounced when the antigen was measured in milk. These results demonstrate that α-la was more heat stable when heated in milk or MUF than when heated in NaCl or KCl, as long as the

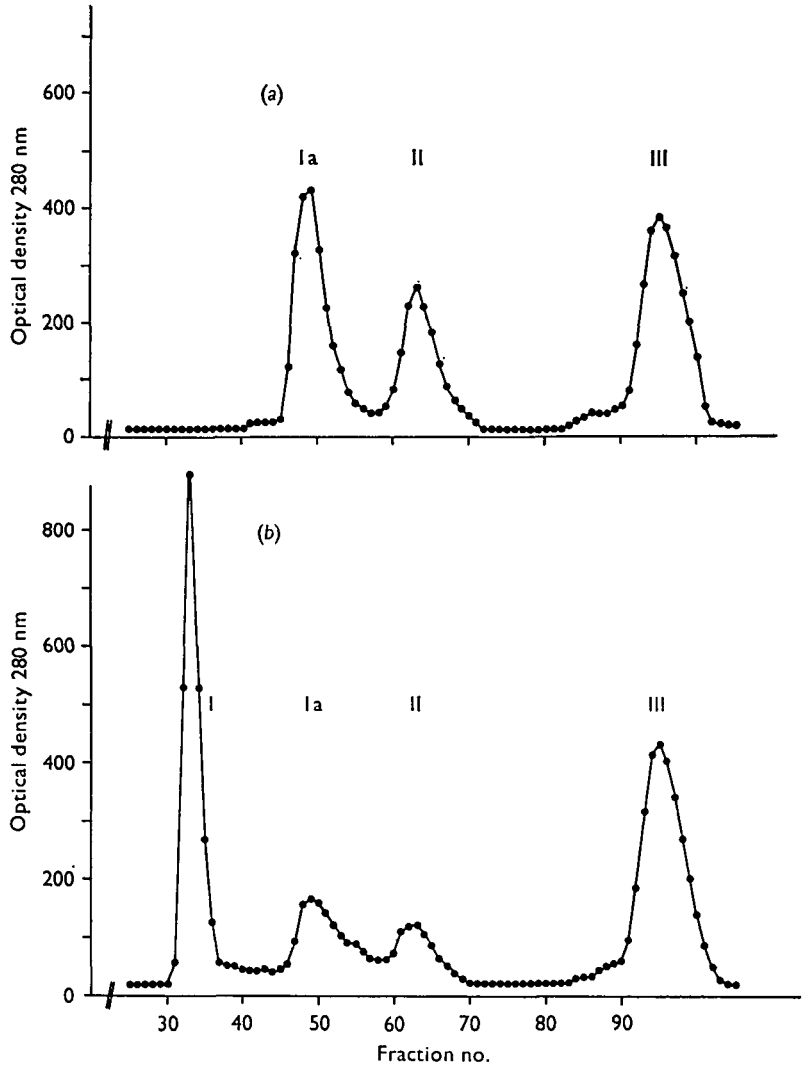

Fig. 3. Chromatography of a solution containing 0·3 % (w/v) of β-lactoglobulin (β-lg) and 0·1 % (w/v) of α-lactalbumin (α-la) dissolved in milk ultrafiltrate (*a*) before and (*b*) after heat treatment for 30 min at 80°. Chromatogram (*a*): peak Ia corresponds to the elution of β-lg, peak II corresponds to the elution of α-la. Peak III is due to absorbing material at 280 nm of milk ultrafiltrate. Chromatogram (*b*): In peak I, only β-lg has serological activity. Conditions of chromatography: column 1·2 × 100 cm of Ultrogel AcA 54. Elution, 0·05 M-phosphate buffer, pH 6·6 containing 0·2 M-NaCl. Rate of elution, 40 ml/h.

temperature of incubation did not exceed 70 °C. Therefore, MUF was used for further studies.

Reversibility of the reactions

To investigate a possible reversibility of the denaturation process, α-la, heated in MUF at 80 °C for 2 h, was allowed to stand at 0 °C or at room temperature for times ranging from 5 min to 3 h, then the complement assay was performed. The percentage of complement fixation was not higher than that usually found in the assay. Thus,

if a partial renaturation influencing the serological properties of the molecule does occur, it must happen during the short time of the antigen dilution and its reaction with the antibody.

Heat-induced interaction of α-lactalbumin and β-lactoglobulin

The discrepancy between the results obtained with α-la heated in milk or in MUF at temperatures over 70 °C was investigated by adding various milk proteins separately or together to the MUF at the concentrations found in milk, and heating the mixtures at 80 °C for 30 min. Fig. 2 (*a*, *b*) shows that when immunoglobulins, bovine serum albumin, lactoferrin and whole casein were all heated together with α-la, no modification of the complement fixation curve appeared, compared with that obtained with α-la alone. However, in the presence of β-lg there was extensive modification of the immunochemical behaviour of α-la (Fig. 2*c*). The peak of the complement fixation curve was shifted toward the region of more antigen with a small diminution in the peak height. This shift was similar to that observed when milk was heated (Fig. 2*d*). It was also found that an increase in Ca concentration up to 0·14 % (w/v) did not modify the immunogenic reaction with α-la.

The modification of antigenicity of α-la due to β-lg could be explained in 2 possible ways: firstly, assuming that a complex of the 2 proteins (as described by Hunziker & Tarassuk, 1965) is formed in our experimental conditions, several antigenic determinants of α-la may have been modified, or partly hidden by their binding with β-lg, decreasing thus the complementarity between the antigen and its specific antibody; in that case, the amount of antigen required for maximum complement fixation would be increased. Secondly, only a part of the available molecules of α-la may have been complexed with β-lg and the conformation of the α-la involved may have been so drastically changed that it would no longer display any reactivity with its homologous antiserum. In this case, the lateral displacement of the complement fixation curve would be a direct measure of the concentration of the uncomplexed α-la remaining in the reaction mixture. However, it is also necessary that the decrease in peak height observed must correspond exactly to the decrease measured with α-la heated alone (compare Fig. 2*a*, *c*).

To test which part of the alternative was correct, a solution of α-la and β-lg mixed at the same ratio as in milk was heated and then filtered on a column of Ultrogel AcA 54. From the chromatogram obtained, a new peak was observed (peak I, Fig. 3*b*), eluted with the void volume of the column, and compared with the chromatogram of the unheated mixture (Fig. 3*a*). Serological activity of α-la was found only in peak II, corresponding to the native protein. It was determined both by the double diffusion technique and the micro-complement fixation which gave identical results. However, a similar experiment with ^{125}I-labelled α-la showed the presence of radioactivity in the new peak, thus indicating the presence of immunologically inactive α-la. In contrast, serological activity of β-lg was found in this peak as well as in the one corresponding to the native protein (peak I*a*). The curve of complement fixation obtained with the protein contained in peak II (Fig. 3*b*) could be superimposed on the one measured with α-la heated alone. Therefore, the heat denaturation of the uncomplexed α-la was identical with that observed with the pure heated protein. The shifting of the complement fixation curve observed with heated α-la in presence of

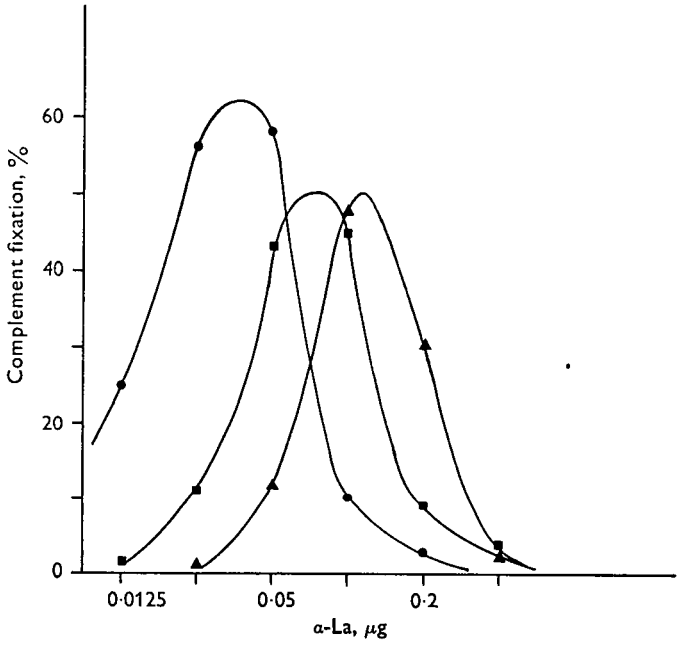

Fig. 4. Influence of β-lactoglobulin (β-lg) on the serological properties of α-lactalbumin (α-la) during heat treatment for 30 min at 80 °C. ●, α-La and β-lg unheated; ▲, α-la and β-lg heated together; ■, α-la heated alone, then mixed with β-lg also heated alone (both proteins were cooled before being mixed). Protein concentrations 0·3 % (w/v) and 0·1 % (w/v) for β-lg and α-la respectively.

β-lg, or in milk, was produced by the loss of a certain amount of antigen complexed with β-lg.

Conditions of complex formation between α-lactalbumin and β-lactoglobulin

In their studies on the heat-induced interactions between κ-casein and β-lg Zittle *et al.* (1962) showed that it was not necessary to heat the 2 proteins together to obtain a complex; when β-lg was heated alone and then mixed with unheated κ-casein, a complex formed.

Our results showed that heat-induced structural modification of α-la as well as of β-lg was necessary to form a complex between the 2 proteins (Fig. 4). However, when these proteins were heated separately and then mixed, the amount of α-la bound to β-lg was smaller than that obtained when the 2 proteins were heated together (Fig. 4, smaller shift toward the region of more antigen). Thus, less β-lg was involved in the complex, probably because of a partial renaturation of the cooled protein, as described by Dupont (1965) for the A variant.

Influence of the molar ratio between β-lactoglobulin and α-lactalbumin on the serological behaviour of α-lactalbumin.

Mixtures containing a constant amount of α-la (1·0 mg/ml) and various concentrations of β-lg were heated at 80 °C for different times, and the serological activity of α-la measured. Two distinct phases were observed in the course of the

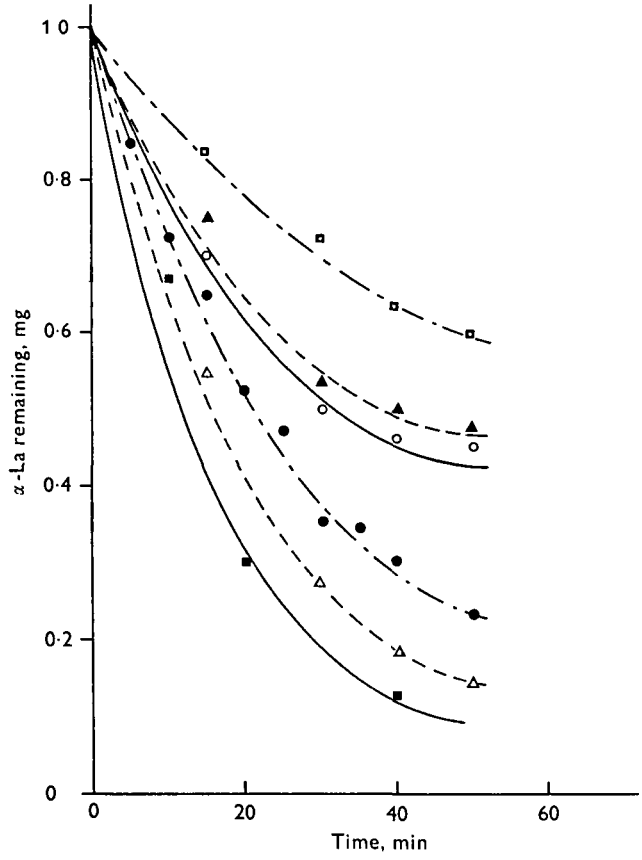

Fig. 5. Rate of formation of the heat-induced complex of α-lactalbumin (α-la) with β-lactoglobulin (β-lg) at different concentrations of β-lg. Mixture dissolved in milk ultrafiltrate containing a constant amount of α-la (1·0 mg/ml), but various concentrations of β-lg were heated at 80 °C for the indicated times. The molar ratios of β-lg to α-la were 2·23 (■), 1·25 (raw milk) (△), 0·98 (●), 0·75 (○), 0·50 (▲), 0·25 (□).

modification of the serological behaviour of α-la. In the first 30–40 min heating there was a progressive shift of the complement fixation curves toward the region of higher antigen concentration. By plotting the residual amount of α-la (calculated from the successive shifts) as a function of time, it was observed that the first phase of the reaction followed an exponential rate; the higher the molar ratio between both proteins, the lower the amount of uncomplexed α-la (Fig. 5). After 40–50 min incubation, the lateral shifting did not proceed further, but only a diminution of the peak height was observed, at a constant amount of antigen. By plotting the remaining percentage of complement fixed during the second phase as a function of time, the experimental values obtained with α-la heated alone were found to lie on an exponential curve (Fig. 6). The experimental values found with the other molar ratios fell on the same curve.

These results demonstrate that α-la undergoes at least 2 types of modification in the first phase of the reaction. There is first a heat-induced structural change of the molecule which is indispensable for the complex formation with heat-modified β-lg

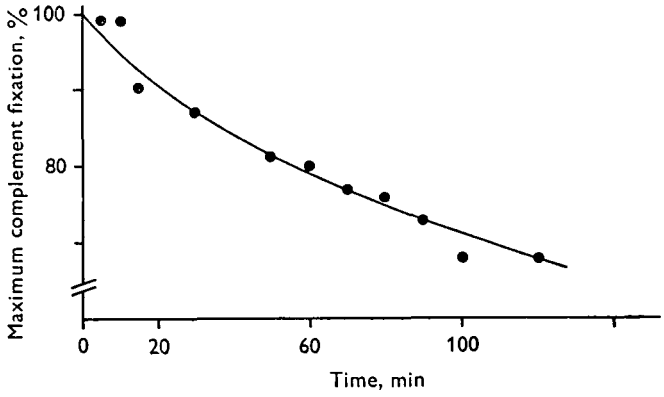

Fig. 6. Rate of heat-induced decrease of the complement fixing activity of α-lactalbumin heated alone. The percentage of maximum fixation was extrapolated from the percentage of complement fixed by the native protein. The experimental values found with the other molar ratios, after the complex formation with β-lactoglobulin had ceased, fell on the same curve.

which follows. Once the reaction between α-la and β-lg has ceased, the second phase seems to correspond to the continuing structural modification of uncomplexed α-la. In the first phase of the reaction (Fig. 5) the maximum number of molecules of α-la reacting with β-lg is reached in milk (molar ratio of about 1·2), since the addition of an excess of β-lg up to a ratio of 2·2 did not markedly increase the amount of complex formation. This is in accordance with the results of Lyster (1970). By decreasing the molar ratio to 0·98, 0·75, 0·50 or 0·25, the amount of α-la remaining uncomplexed increased. However, the complex formation between the 2 proteins is not a linear function of the molar ratios.

Kinetics of the reactions

The kinetics of structural modification of heated α-la were studied, to compare our results with those of Lyster (1970), using the experimental points given in Fig. 6. After 15 min incubation at 80 °C, they lie on the curve obtained from the equation for first-order kinetics,

$$-dc/dt = k_1 c, \tag{1}$$

where c is the concentration of native α-la at time t, and k_1 the kinetic constant of the reaction. The value of k_1 was determined from the integrated and rearranged equation,

$$-k_1 t = 2{\cdot}303 \log c/c_0, \tag{2}$$

where c_0 is the initial concentration of the protein, by plotting $\log c/c_0$ as a function of time and measuring the slope of the line drawn, as shown in Fig. 7. The value thus obtained for the kinetic constant was

$$k_1 = 4{\cdot}8 \times 10^{-5}\ \mathrm{s}^{-1}.$$

It was not possible to determine a change of the protein structure during the first 10 min incubation; after 15 min, a pronounced structural modification was observed,

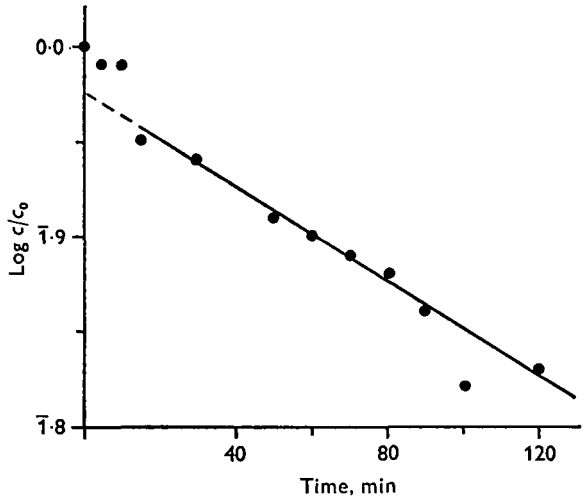

Fig. 7. First-order reaction plot for the structural modification of α-lactalbumin (1·0 mg/ml), heated in milk ultrafiltrate at 80 °C. The experimental points of Fig. 6 were used. For c/c_0 see equation (1) and (2) in text.

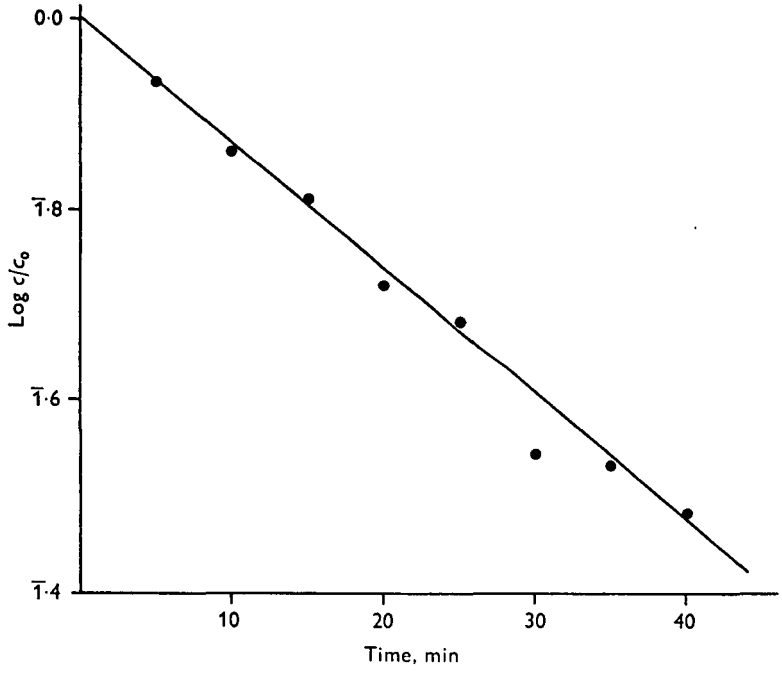

Fig. 8. First-order reaction plot for the complex formation of α-lactalbumin (α-la) with β-lactoglobulin (β-lg) in milk ultrafiltrate, at 80 °C. Molar ratio of β-lg to α-la, 0·98. The experimenta points of Fig. 5 were used. For c/c_0 see equations (1) and (2) in text.

whose rate follows first-order kinetics. However, the reaction is certainly more complex than a simple 2-state type reaction (native and denatured molecules), and involves probably successive discrete structural modifications. We consider that the heat-induced conformational change of α-la incubated at 80 °C is a pseudo first-order reaction.

The kinetics of reaction of α-la with β-lg for the first 40 min heating at 80 °C has also been studied. The experimental points given in Fig. 5 for a molar ratio of 1·0 have been used, and equation (2) for first-order kinetics applied. The value obtained for the kinetic constant k_2, calculated from the slope of the line drawn in Fig. 8, was

$$k_2 = 5{\cdot}12 \times 10^{-4}\,\mathrm{s}^{-1}.$$

This value can be compared with the value of k_2 calculated from the general equation established by Lyster (1970) for the denaturation of α-la in skim-milk,

$$\log k_2 = 7{\cdot}15 - 3{\cdot}60\ (10^3/T). \tag{3}$$

The value thus obtained at 80 °C would be

$$k_2 = 8{\cdot}95 \times 10^{-4}\,\mathrm{s}^{-1}.$$

The 2 values of k_2 are of the same order of magnitude; the small discrepancy may be due to the inaccuracy of equation (3) at this temperature, since it is applicable to temperatures between 90 and 155 °C. The molar ratio between α-la and β-lg in skim-milk would also differ from the value of 1·0, as used under our experimental conditions.

One would have expected the reaction to have second-order kinetics, since our results have shown unequivocally the dependence of the heat-induced modification of α-la on the concentration of β-lg. However, the experimental values cannot be described by the equation for a second-order reaction. Such a contradiction may be explained by invoking a complex procedure of structural modification of the protein, involving consecutive, partly reversible initial stages which may involve β-lg and be rate determining. There was no measurable structural change in α-la heated alone during the first 10 min incubation, and the rate of the reaction was slow, when compared with the rate of the complex formation with β-lg. Thus, the structural modification of α-la necessary for the complex formation with β-lg is apparently not the same as that found when the protein is heated alone. Therefore, there must be at least one intermediate step in the conformational change, which cannot be observed with our method. Such a thermal transition has been described by Takesada, Nakanishi & Tsuboi (1973) for α-la dissolved in D_2O, pH 5·7, at 58 °C; these results have been confirmed in our laboratories for the protein dissolved in a synthetic milk-ultrafiltrate, pH 6·8, at 62 °C (Dr Rüegg, personal communication).

Influence of α-lactalbumin on the complex formation between β-lactoglobulin and κ-casein

Even though the heat-induced interaction between β-lg and κ-casein is a well-known phenomenon (for a review, see Sawyer, 1969), a possible influence of α-la on this complex formation has never been demonstrated. Since we have shown that κ-casein does not modify the serological activity of α-la complexed with β-lg, it is conceivable that α-la affects the serological properties of β-lg under these conditions. For these reasons, we have investigated the serological behaviour of β-lg by the method of micro complement fixation, using an antiserum against this protein.

The results (Fig. 9), show that after 30 min of heating β-lg with α-la, about 8 times more antigen was required for maximum complement fixation, as compared with the antigen concentration needed for maximum fixation with the native protein. When β-lg was incubated with κ-casein, 6 times more antigen was needed for maximum

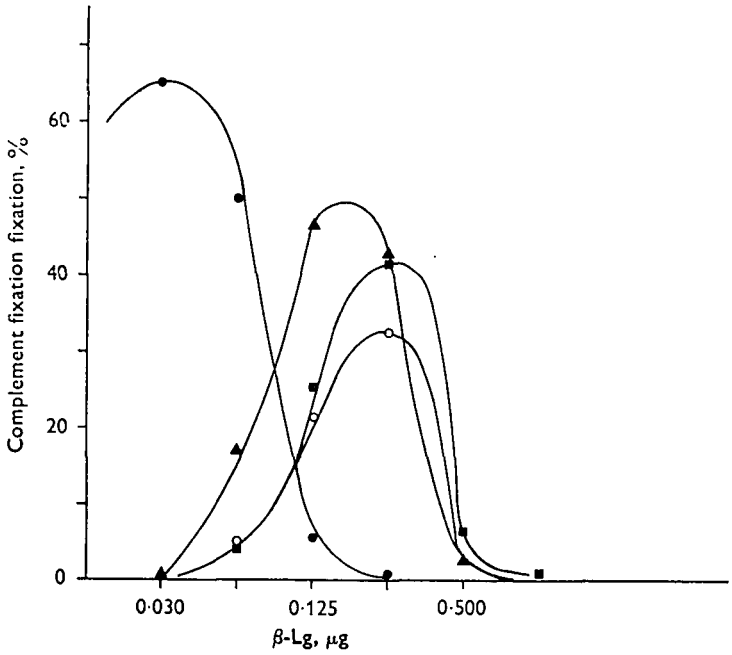

Fig. 9. Influence of α-lactalbumin (α-la) on the heat-induced complex formation between β-lactoglobulin (β-lg) and κ-casein, as measured by the modification of the serological properties of β-lg. ●, β-Lg alone; ▲, β-lg and α-la heated together at 80 °C for 30 min; ■, β-lg heated with α-la and κ-casein; ○, β-lg heated with κ-casein.

fixation. In both experiments, the lateral displacement was accompanied by a significant vertical shift, which was more pronounced with β-lg heated with κ-casein than when incubated with α-la. The addition of α-la with κ-casein modified the serological behaviour of β-lg in the same way as κ-casein did, since one needed an identical amount of antigen concentration for maximum complement fixation. However, the amount of complement fixed was higher by about 15 %. Therefore, the molecular conformation of β-lg complexed with κ-casein was not much influenced by the simultaneous formation of another complex with α-la. There was also a complete lack of influence of κ-casein on the serological activity of α-la complexed with β-lg. A marked difference was observed between the complement fixation curve obtained with β-lg heated in the presence of α-la or in the presence of κ-casein (Fig. 9). These results indicate that both proteins are bound to distinctly different parts of the β-lg molecule, or to 2 different types of heat-modified molecules, e.g. monomers or polymers.

By using micro complement fixation, it was possible to determine the phenomenon responsible for most of the loss of the serological activity of α-la in heated milk: a complex formation occurs between β-lg and α-la.

This type of experiment should allow a better understanding of the changes in the properties of milk occurring during heat treatment. By measuring quantitatively and specifically the alteration of the serological properties of a whey protein in heated milk, and by comparing the results with those obtained in isolated systems, it is possible to study accurately the phenomena occurring in whole milk.

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